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Rat Liver Cytochrome P450-mediated Metabolic Activation of Methoxsalen and Structurally Related Compounds and its Relation to Enzyme Inhibition

DAVID J. WILKINSON AND JEFFREY R. FRY

Department of Physiology and Pharmacology, Medical School, Queens Medical Centre, Nottingham NG7 2UH, UK

Abstract

The metabolic activation and enzyme inhibition characteristics of methoxsalen were investigated in rat liver microsomes obtained from untreated animals and those treated with a number of prototypic inducers of cytochrome P450.

Glutathione depletion assays have been carried out which show reactive metabolite generation to be markedly increased following phenobarbitone and β-naphthoflavone induction. Moreover, isoniazid induction led to levels of glutathione depletion significantly higher than those seen with other forms of induction, suggesting an important role for the cytochrome P4502E1 isozyme in the metabolic activation process.

Methoxsalen was shown to be an extremely potent inhibitor of 7-ethoxycoumarin-*O*-de-ethylase activity, with inhibition constants of the order of 5 μM with microsomes obtained from untreated, phenobarbitone- and β-naphthoflavone-induced animals. In contrast, constants obtained with microsomes obtained from isoniazid-induced animals were found to be markedly higher.

Comparisons of the inhibition of 7-ethoxy and 7-pentoxyresorufin-*O*-dealkylase activities by methoxsalen and a number of structurally-related compounds have shown that a complete tricyclic ring system and an unsaturated 4′,5′-bond are structural prerequisites in the formation of reactive metabolites which inhibit cytochrome P450. These data implicate the furan ring system as the source of these metabolites and rule out the involvement of the pyrone ring system in the inhibition process.

Methoxsalen (8-methoxypsoralen, xanthotoxin; Fig. 1) is a naturally-occurring linear furocoumarin derivative, found predominantly in plants of the umbelliferae and rutaceae orders, and in several edible species such as celery, parsnips, limes and figs (Pathak et al 1962; Ivie et al 1981). Many psoralens, and in particular methoxsalen, show potent photosensitizing properties which have been harnessed by man in the treatment, or photochemotherapy, of a number of dermatological disorders such as psoriasis (Parrish et al 1974), vitiligo (Parrish et al 1976), and cutaneous T-cell lymphoma (Gilchrest et al 1976).

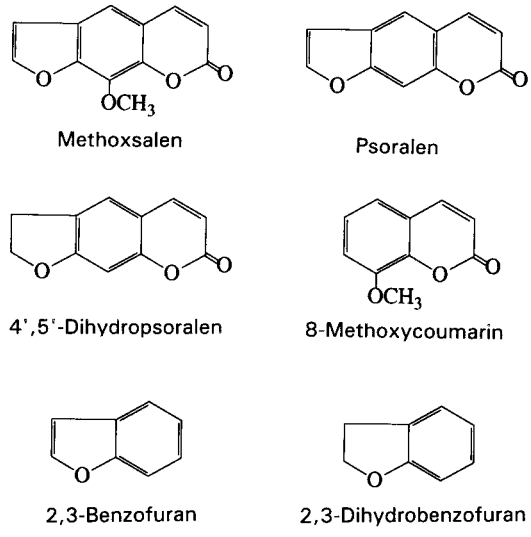

FIG. 1. Chemical structures of methoxsalen and other compounds under study.

Correspondence: J. R. Fry, Department of Physiology and Pharmacology, Medical School, Queens Medical Centre, Nottingham NG7 2UH, UK.

Earlier work has shown that methoxsalen is metabolically activated by rat and human liver cytochrome P450 to metabolites which irreversibly bind to microsomal protein and inactivate P450 (Tinel et al 1987; Labbe et al 1989). The potency of this suicide inhibition has been shown, under some circumstances, to be much greater than that of some classical P450 inhibitors; indeed methoxsalen has produced significant inhibition at concentrations at which SKF 525-A and piperonyl butoxide had no effect (Letterton et al 1986; Tinel et al 1987). These studies have also investigated the inactivation of P450 by various psoralen derivatives and have suggested that the furan ring moiety of the psoralen molecule is likely to be the origin of the reactive metabolite responsible for the inhibition. This theory was based upon the fact that methoxsalen, 5-methoxypsoralen and psoralen itself all produced comparable levels of P450 inhibition, whereas 4,5′,8-trimethylpsoralen, with the outer furan double bond blocked by a methyl group, had a negligible effect. What remains unclear, however, is the role of pyrone ring metabolites in the metabolic activation or inhibition process, as the 3,4-double bond of trimethylpsoralen is also blocked by a methyl

group. Metabolism studies have identified both furan and pyrone ring-opened urinary metabolites of methoxsalen, formed, presumably, following P450-mediated oxidative attack on each ring (Mays et al 1986). Earlier work within this laboratory has identified reactive, hepatotoxic metabolites of coumarin, formed following an initial 3,4-epoxidation, with subsequent ring opening (Fentem et al 1991). It is conceivable, therefore, that a pyrone or furan ring-originated metabolite may produce the P450 inhibition seen with methoxsalen.

The present studies have, therefore, further investigated the activation and inhibition processes, with the aims being to identify P450 isozyme specificity and the nature of the inhibitory metabolites.

Materials and Methods

Chemicals

The chemical structures of the compounds under study are shown in Fig. 1. Methoxsalen was purchased from Sigma Chemical Co. Ltd, Poole, UK. Psoralen was obtained from Fluka Chemicals Ltd, Gillingham, UK. 2,3-Benzofuran was purchased from Aldrich Chemical Co. Ltd, Gillingham, UK. 2,3-Dihydrobenzofuran was obtained from Janssen Chimica, Hyde, UK. 4′,5′-Dihydropsoralen was a very generous gift from Dr H. K. Kang, Sunchon National University, Korea. 8-Methoxycoumarin was prepared to high purity by methylation of 8-hydroxycoumarin, synthesized by the method of Murayama et al (1972). 7-Ethoxycoumarin was synthesized according to the method of Ullrich & Weber (1972). Ethoxy- and pentoxyresorufins were synthesized by the methods of Prough et al (1978) and Burke & Mayer (1983), respectively. Characterization and purity determination of the synthesized compounds was carried out using thin layer chromatography (TLC), measurements of melting point, and in the case of 8-methoxycoumarin, mass spectroscopy. TLC analysis revealed the existence of single spots for each synthesized material, and melting points were within the ranges quoted in the original publications.

Animals and pretreatment

Male Wistar rats (Sutton Bonington strain, approx. 150 g) were obtained from the University of Nottingham Medical School Animal Unit, and were allowed free access to food and water. Animals were pretreated with sodium phenobarbitone and isoniazid for 7 and 10 days, respectively, as a 0·1% (w/v) solution in the drinking water. β-Naphthoflavone was administered over three days at a dose of 80 mg kg^{-1} via intraperitoneal injection in arachis oil. Control animals received no treatment. Animals were killed by cervical dislocation 24 h after the final treatment.

Preparation of liver microsomes

Microsomal fractions were prepared from the pooled livers of six animals by the calcium aggregation method of Kamath & Narayan (1972). Protein content was measured by the method of Lowry et al (1951), and P450 content by the method of Omura & Sato (1964). Microsomes were stored at −180°C.

Glutathione-depletion assay

Formation of reactive metabolites was detected from the depletion of a known amount of exogenous glutathione in the assay described by Garle & Fry (1989). Test compounds were dissolved in *N,N*-dimethylformamide and added at concentrations of 0·005–1·0 mM, with a glutathione concentration of 200 μM. Incubations were carried out with or without an isocitrate-based NADPH-generating system, and terminated at 30 min, with the glutathione remaining determined spectrophotometrically following reaction with 5′,5′-dithio-bis-(nitrobenzoic acid). In time course experiments, incubations were terminated at various time intervals of up to 30 min.

7-Ethoxycoumarin-O-de-ethylase (ECOD) activity

The inhibition of ECOD activity by methoxsalen was determined according to the method described by Paterson et al (1984). After the initial 2-min preincubation stage, microsomes were incubated with methoxsalen (5–25 μM) for a further 2 min before the addition of 7-ethoxycoumarin. Incubations were terminated after a further 10-min incubation and fluorescence of an alkaline extract was then determined using a Baird RC200 Ratiometric fluorimeter.

Ethoxy- and pentoxyresorufin-O-de-alkylase activities (EROD and PROD)

EROD and PROD activities were determined by the method described by Prough et al (1978), with test compounds (5–25 μM) added half way through the initial 10 min preincubation stage. Fluorescence was measured against a Rhodamine B standard.

Statistical analysis of data

Variance in the glutathione depletion was initially confirmed using a one-way analysis of variance test, and the location of this variance was determined using the Dunnett procedure which provided a confidence level for the difference between each treatment mean and the control mean. Significance was assigned for $P < 0.05$.

Results

Reactive metabolite formation

Fig. 2 represents the glutathione-depletion profile of methoxsalen obtained with microsomes isolated from untreated rats and those treated with phenobarbitone, β-naphthoflavone and isoniazid. P450 induction due to these compounds led to dose-dependent increases in glutathione depletion, reaching maximum values significantly greater than those obtained with control microsomes. Isoniazid treatment produced the greatest depletion, with a peak of approximately 30 nmol/30 min (mg protein)$^{-1}$, being some 29% higher than that obtained with phenobarbitone induction. In the absence of cofactors, and hence an NADPH source, very little activity was measureable. A notable trend in the data obtained with microsomes obtained from untreated animals and those induced with phenobarbitone and isoniazid was that maximum depletion occurred at a methoxsalen concentration of 0·5 mM. The glutathione-depletion data for the other compounds under study has thus been presented at this concentration, as all

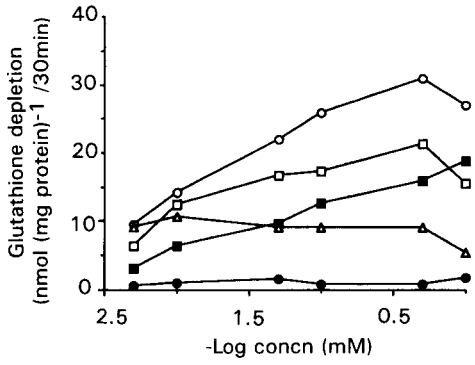

FIG. 2. Methoxsalen-mediated glutathione depletion in rat liver microsomes obtained from untreated animals (△), and those treated with isoniazid (○), phenobarbitone (□) and β-naphthoflavone, both in the presence (■) and absence (●) of an NADPH-generating system. The data are shown as means of at least four experiments. Error bars are omitted for clarity.

trends observable over the complete concentration range are still apparent at this single concentration. These data are indicated in Table 1.

Psoralen was found to behave in a similar manner to methoxsalen in that dose-dependent increases in glutathione depletion were produced. However, the major difference was that induction with phenobarbitone and β-naphthoflavone led to depletion values significantly lower than those of the controls. With isoniazid induction, however, the differences in depletion compared with controls were insignificant. Again, in the absence of cofactors, very little activity was found. 4′,5′-Dihydropsoralen was found to be a very poor depletor of glutathione, with values < 4·0 nmol/30 min (mg protein)$^{-1}$ in all cases.

The bicyclic compounds under study were found to behave rather differently to the three psoralens. 8-Methoxycoumarin showed marginal amounts of depletion with microsomes obtained from untreated and from isoniazid-induced animals, whereas induction with phenobarbitone or β-naphthoflavone led to levels of depletion significantly greater than seen with the psoralens. 2,3-Benzofuran was the most potent compound tested in the glutathione-depletion assay, and its performance relative to that of 2,3-dihydrobenzofuran demonstrates the importance of the 2,3-double bond in the metabolic activation process.

Time course of glutathione depletion

Fig. 3 represents the time courses of methoxsalen-induced glutathione depletion mediated by 1·0 mM methoxsalen using liver microsomes isolated from rats treated with β-naphthoflavone or isoniazid. The glutathione-depleting characteristics of methoxsalen were compared with those of an equimolar amount of coumarin, which is also metabolically activated to reactive metabolites but does not inhibit P450 to any appreciable extent.

With β-naphthoflavone induction, methoxsalen produced a glutathione-depletion profile which displayed a saturation effect after 10–15 min, in marked contrast to that obtained for coumarin, which generated a time-dependent depletion profile, with the maximum at 30 min sixfold greater. Isoniazid induction, however, produced a quite different effect with respect to methoxsalen. Whilst coumarin again produced a time dependent increase in glutathione depletion, methoxsalen showed an essentially identical trend, with no indication of the plateau effect seen with β-naphthoflavone induction.

Inhibition of ECOD activity

ECOD activities were measured in incubations carried out in the presence of methoxsalen at concentrations of 5 to 25 μM, with 7-ethoxycoumarin concentrations of 5 and 20 μM. In all cases, dose-dependent decreases in the enzyme activity were noted. The data obtained were plotted in the form of Dixon plots, from which enzyme inhibition constants (K_i values) were determined. These constants, and the nature of the inhibition generated, are indicated in Table 2. With microsomes obtained from untreated and phenobarbitone- and β-naphthoflavone-treated animals, the K_i values measured were extremely small at 2·6–6·4 μM, indicative of very potent inhibition of the enzyme. The constant obtained with isoniazid induction, however, was markedly higher at 46 μM. Methoxsalen acted as a competitive inhibitor of ECOD, with the exception of phenobarbitone-treated rats, for which methoxsalen acted as a non-competitive inhibitor.

Inhibition of EROD and PROD activities

The ability of the six test compounds to inhibit EROD and PROD activities was investigated using liver microsomes, which generated the highest enzyme activities, i.e. from β-naphthoflavone- and phenobarbitone-induced animals,

Table 1. Glutathione depletion mediated by methoxsalen and related compounds at 0·5 mM. All values are expressed as nmol depleted (mg protein)$^{-1}$/30 min (means ± s.d.).

Substrate	Treatment			
	Control	Isoniazid	Phenobarbitone	β-Naphthoflavone
Methoxsalen	9·1 ± 1.5	30·2 ± 2·8*	21·5 ± 1·0*	15·7 ± 1·7* (0·73 ± 0·3)
Psoralen	18·2 ± 1·5 (1·0 ± 0·8)	20·2 ± 0·3	6·8 ± 1·0*	12·7 ± 5·7*
8-Methoxycoumarin	3·3 ± 2·7	9·8 ± 4·5*	30·4 ± 8·9*	50·2 ± 4·1*
2,3-Benzofuran	24·6 ± 2·6 (1·9 ± 0·9)	30·2 ± 1·6*	90·3 ± 2·9* (0·11 ± 0·05)	55·0 ± 3·3*
2,3-Dihydrobenzofuran	2·3 ± 1·6	3·2 ± 2·2	38·2 ± 9·8*	13·3 ± 1·1*
4′,5′-Dihydropsoralen	ND	3·7 ± 0·2	3·7 ± 0·8	3·2 ± 0·2

Values in parentheses indicate data obtained in the absence of an NADPH-generating system. ND indicates value not determined. *$P < 0·05$ compared with control.

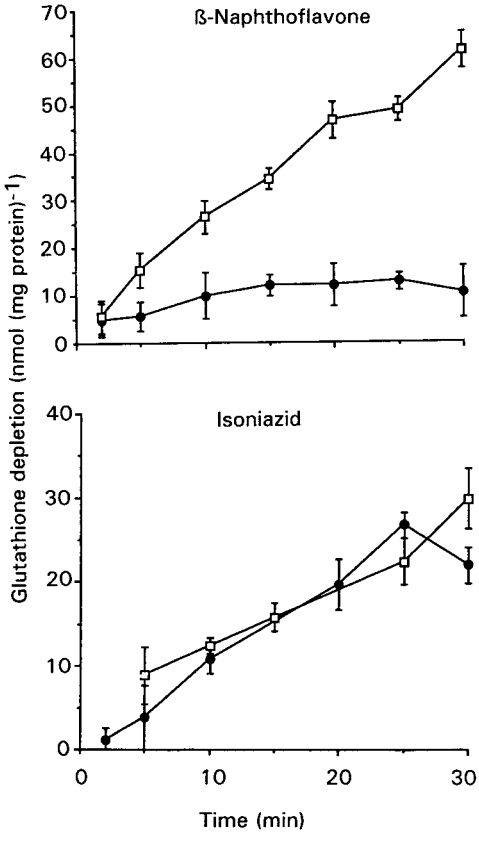

FIG. 3. Time courses of the glutathione depletion mediated by 1·0 mM methoxsalen (●) and 1·0 mM coumarin (□) in rat liver microsomes isolated from β-naphthoflavone- and isoniazid-treated animals. Data shown as means ± s.d. from at least four experiments.

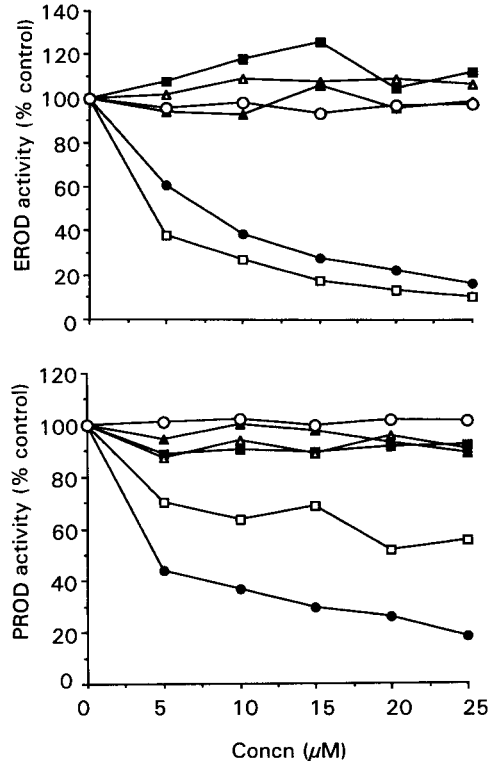

FIG. 4. Inhibition of EROD and PROD activities by methoxsalen (●) and related compounds. Psoralen (□), 4′,5′-dihydropsoralen (■), 8-methoxycoumarin (△), 2,3-benzofuran (▲) and 2,3-dihydrobenzofuran (○). Data shown as mean values from at least four experiments. Error bars are omitted for clarity.

respectively. Liver microsomes isolated from animals treated with other inducers were found to show very similar trends (data not shown). It is very clear from Fig. 4 that only methoxsalen and psoralen had the capability to inhibit both enzymes. In the case of EROD, both methoxsalen and psoralen produced IC50 values of 5–10 μM, as did methoxsalen in the assay of PROD activity. In the latter assay, psoralen was found to be less effective, the reason for which is unknown. 4′,5′-Dihydropsoralen and the three bicyclic analogues showed little or no evidence of being inhibitors of these particular mono-oxygenase activities.

Discussion

Methoxsalen possesses two centres at which reactive electrophilic metabolites may be produced: the 4′,5′-double bond on the furan ring (by analogy with reactive furans such as aflatoxin B_1 and 4-ipomeanol), and the 3,4-double bond on the pyrone ring (by analogy with coumarin and the precocenes). Consistent with this, ring-opened metabolites derived from both rings have been identified as metabolites of methoxsalen in-vivo, in animals and in man (Kolis et al 1979; Schmid et al 1980; Mays et al 1986), these most likely having arisen from oxidative attack at the respective double bonds. In this study we have attempted to determine the relative importance of these sites of attack to the interaction of methoxsalen with cytochrome P450.

Table 2. Methoxsalen-enzyme inhibition constants and nature of ECOD inhibition.

Treatment	K_i (μM)	Nature of inhibition
Untreated	5·1	Competitive
β-Naphthoflavone	2·6	Competitive
Phenobarbitone	6·4	Non-competitive
Isoniazid	46·0	Competitive

Methoxsalen was demonstrated to be a potent inhibitor of liver P450-mediated mono-oxygenase activity, this being in agreement with previous observations. In addition, the potent inhibition in liver microsomes isolated from untreated rats (which express constitutive P450 forms) and from rats treated with β-naphthoflavone (enriched in P4501A forms) and phenobarbitone (enriched in P4502B forms) indicates that methoxsalen is active towards a number of P450 forms. Psoralen was a less potent inhibitor of P450-mediated activity. The complete tricyclic structure of this group of compounds was required for this inhibitory action, as was the unsaturated 4′,5′-bond, as judged by the ineffectiveness of the bicyclic compounds and the saturated 4′,5′-dihydropsoralen.

We have previously demonstrated that the glutathione-depletion assay used in this study provides a reliable index of P450-mediated generation of reactive electrophiles, and good positive responses were obtained with furans

(4-ipomeanol) and pyrones (coumarin) (Garle & Fry 1989, 1990). Glutathione depletion caused by methoxsalen and psoralen (this study) were considerably less than that reported for 4-ipomeanol and coumarin. We consider it likely that this lower reactivity is related to the P450-inhibitory effects of these compounds. Thus, the non-inhibitory bicyclic compounds were more effective than methoxsalen or psoralen as depletors of glutathione, and the highest levels of reactive metabolite generation from methoxsalen occurred with liver microsomes (isoniazid-treated animals) that were characterized by a lower sensitivity to methoxsalen inhibition. In addition, the low K_i and IC50 values obtained for methoxsalen indicate avid binding to P450. Taken together, these data support the proposal that methoxsalen metabolites, upon formation, immediately, and tightly, bind to, and destroy, P450, so limiting the amount of metabolites available for conjugation with glutathione (Fouin-Fortunet et al 1986). The production of reactive electrophiles from the psoralens required the presence of the 4′,5′-double bond; 4′,5′-dihydropsoralen showed very little activity in this assay.

As described above, methoxsalen bears structural motifs which are similar to those encountered in established hepatotoxins such as aflatoxin B_1 and coumarin. In addition, high levels of methoxsalen metabolites bind to tissue macromolecules (Mays et al 1989) in a manner analogous to hepatotoxins such as paracetamol (Vermeulen et al 1992). However, there is little evidence to indicate that methoxsalen administration generates hepatotoxicity in man, and on-going studies in this laboratory suggest that the compound is of low toxicity to rat hepatocytes (D. J. Wilkinson, unpublished data). Nevertheless, various reports of hepatotoxicity to rodents and primates on administration of methoxsalen in high doses have been published (Hakim et al 1961; Dunnick et al 1984; Rozman et al 1989). A possible explanation for this low toxicity in the face of demonstrable reactive metabolite production, is that metabolites of methoxsalen, once generated, are avidly bound within the P450 environment and cannot escape to initiate tissue damage.

Isoniazid was used as a prototypic inducer of P4502E1 (Koop et al 1985). Methoxsalen was significantly less potent as an inhibitor of P450-mediated activity in liver microsomes from isoniazid-treated rats, whilst the highest level of reactive metabolite generation from methoxsalen was recorded from these microsomes. These data suggest that the 2E1 isoform has some structural or conformational property which renders it less sensitive to inhibition by methoxsalen metabolites. This proposal is consistent with the markedly different time-courses of glutathione depletion illustrated in Fig. 3. This proposal may also explain the glutathione-depletion profiles obtained with psoralen.

The glutathione-depletion data with liver microsomes from isoniazid-treated rats may also point to a role for P4502E1 in the bioactivation of methoxsalen. In agreement with this, previous investigations have demonstrated that ethanol (also a P4502E1 inducer) produced a strong inducing effect on the metabolism of methoxsalen by yeast (Pognon et al 1984). Further studies are required to establish the role of P4502E1 in methoxsalen activation.

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